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The Journey to Fielded BioInstrumentation

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The Journey to Fielded BioInstrumentation

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Abstract

Over the last ten years, a team at Lawrence Livermore National Laboratory created fieldable instruments that performed identification/quantification via bioassays. These instruments have been based on molecular surface-recognition assays, such as immunoassays, and on nucleic-acid-based assays, such as the polymerase chain reaction. In 1996, we participated in the Joint Field Trials 3, employing both immunoassays as well as the polymerase chair reaction. In 1998, we participated in the Joint Field Trials 4, using only the real-time polymerase chain reaction, as implemented on a 10-chamber instrument. Our hand-held, real-time PCR instrument, known as HANAA has been commercialized as the Bioseeq®, by Smiths Detection. More recently, teams from LLNL have built and fielded an autonomous pathogen detection system (APDS).

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Index Terms – Field Trials, PCR, Flow cytometry, Immunoassay, Autonomous

Introduction

Human beings, both from our desire to survive and also from more heuristic, scientific motivations, have developed numerous techniques to detect, identify, and quantify the presence of biological materials in our environment. Naturally, we are particularly concerned with exposure to possibly-hazardous bacteria, viruses, or any other biomaterials with which we might have contact. When possible, both the culturing of a bacterium in an appropriate medium and the growing of a virus in its host organism provide powerful and sensitive methods that have been used for decades[1] to identify microbes. Unfortunately, these approaches often require time periods of 24 hours or longer. Moreover, many bacterial samples are unculturable, viruses can be difficult to propagate in the laboratory, and a sample that has been exposed to sunlight and/or air may contain only damaged/non-infective viruses[2,3].

Surface molecular-recognition assays such as immunoassays have been used for decades to identify both organisms and toxins[4,5], while PCR-based assays[6-10] have emerged over the past decade and a half as powerful identifiers of organisms[11]. All three approaches are employed, routinely, in clinical and biological laboratories around the world[12-14]. We created a portable, high-performance flow cytometer to perform immunoassays for the laboratory portion of the Joint Field Trials 3 (JFT3), and we created portable instrumentation to perform PCR assays during the JFT3 and the laboratory portion of the Joint Field Trials 4 (JFT4). Based on these successes, we were able to secure support to create a hand-held, 4-chamber, real-time PCR instrument (the hand-held, advanced nucleic-acid analyzer, a.k.a the "HANAA"), as well as a fully autonomous bioaerosol analyzer (the autonomous pathogen detection system, a.k.a the "APDS").

JFT3

As shown in Figure 1, in the fall of 1996 a team of scientists and engineers from LLNL participated in the JFT3, held in the "old" Baker Laboratory of Dugway Proving Grounds, Dugway, UT. We took a flow cytometer for immunoassays (see, Figure 2) and thermal cyclers for PCR assays, which were followed by gel electrophoresis (see, Figure 5). The flow cytometer was based on the flow-stream-waveguide technique, which made it particularly amenable to rough transport, rapid alignment, and field use, has been described elsewhere[15-17]. We also used two types of thermal-cycling instruments (see, below), a Perkin-Elmer 9600™ and the MATCI (miniature analytical thermal-cycling instrument), the latter having been described elsewhere[18,19]. Construction of the MATCI was actually finished after the JFT3 commenced!

For JFT3, unknowns consisted of four possible simulants - the spore-form of the bacterium *Bacillus globigii* (*B.g.*), which is now referred to as *Bacillus atrophaeus*, the vegetative bacterium *Erwinnia herbicola* (*E.h.*), the retrovirus MS2, and the protein ovalbumin. The ranges of concentration for these simulants were 10^3 to 10^6 colony-forming units (cfu)/mL, 10^5 to 10^8 plaque-forming units (pfu)/mL, and 0.1 ng/mL to 100 ng/mL, for bacteria, virus, and protein, respectively.

Immunoassay - Laboratory portion of the JFT3

We derivatized[20,21] the antibodies that had been provided to us by our colleagues from the Naval Medical Research Institute (Drs. Gary Long and Tom O'Brien), producing fluorescein-labeled antibodies for use in the flow cytometer. During JFT3, we used single-target immunoassays, rather than multiplex immunoassays. Each morning of the ten days of trials, we were provided with 36 unknowns. Results had to be submitted at the close of each day for scoring.

For the immunoassays, we divided each unknown into 4 aliquots and added our reagents to each of these aliquots for incubation lasting 20 to 30 minutes at room temperature. Although we had observed that we could reduce the incubation time, there was little motivation to implement this, due to the rapidity of the flow-cytometric assay - typically one minute or less per incubated aliquot. To identify and quantify the bacteria using the flow cytometer, we were able to perform direct detection. That is, we incubated the bacteria with the fluoresceinated antibodies and triggered the flow cytometer on the elastic light scattering from the bacteria particles, themselves. Because the virus and the protein were too small to permit such direct triggering, we relied upon a bead-based "sandwich" assay format, where we linked 5-µm-diameter beads with unlabeled antibodies against either the MS2 or the ovalbumin. This enabled a simulant to bind to its corresponding bead. Next, we added the fluoresceinated antibodies against that simulant and incubated, so that, if the target simulant were present and captured on the derivatized bead, the bead would exhibit fluorescence in the flow cytometer, with the triggering being performed on scattering from the beads. In order to quantify the concentration of the unknowns in our immunoassays for bacteria, Dr. Richard Langlois, who devised and implemented the immunoassays on our flow cytometer, used 10^4/mL of 10-µm latex beads within the reagent solution. Since the

volumetric ratio of reagent solution to sample solution was fixed at 1:1, the known concentration of 10-μm latex beads provided a convenient reference. To quantify the concentration of the viral and protein unknowns, he established “dose-response curves that plotted the fluorescence signal per bead versus concentration of standard solutions of MS2 or ovalbumin.

The direct immumoassay format was particularly effective with the bacterial unknowns, as shown in Figure 3a,b. All concentrations of these unknowns were detected and quantified. Based on these data, we estimated that the detection limit using a high-performance flow cytometer in the direct immunoassay was less than 10^2 cfu/mL. The relatively close values that we measured for each of the four groups of nominal concentrations of the unknowns shows the repeatability of the assay over a two-week period as well as the consistent quality control of those who prepared the unknowns (Drs. Bruce Harper, Doug Winters and their staff at the Dugway Proving Grounds). The data from the sandwich assay on MS2 and ovalbumin are shown in Figure 4a,b. [The magnetic beads that were used in this sandwich assay displayed an autofluorescence that hampered the lower limit of sensitivity.]

Polymerase Chain Reaction - Laboratory portion of the JFT3 and JFT4

Although the miniature analytical thermal-cycling instrument (MATCI) and the commercial thermal-cycling instruments performed as expected during the JFT3, the PCR assays that we used were not fully reliable and yielded erratic data, although the MATCI did perform marginally better than the larger commercial instrument. As part of the original ARPA contract, LLNL provided a MATCI to the Armed Forces Institute of Pathology (AFIP), after the conclusion of the JFT3. Dr. Phillip Belgrader, who ran the forensic laboratory of AFIP, and his colleagues soon learned how to operate the MATCI with excellent results[22].

In 1997, Dr. Belgrader left the AFIP and joined LLNL. In January of 1998, for the laboratory portion of the JFT4, Dr. Belgrader implemented TaqMan® assays that had been developed by our colleagues at the Naval Medical Research Center (Drs. Gary Long and William Nelson). This included the development of a single-tube assay for the retrovirus, MS2. [Dr Belgrader determined that the reverse transcriptase enzyme could function at roughly 40°C to express the target sequence of DNA, and then the first heating to 96°C that commenced the PCR served both to denature the DNA targets and to destroy the reverse transcriptase enzyme, as well.] A description of the PCR instrument and the assays we used at JFT4 has already been published[23,24]. A photograph of some of the LLNL team, taken at Dugway Proving Grounds during the JFT4, is shown in Figure 5.

The challenge materials for the laboratory portion of the JFT4 were the same as those in JFT3, except that the range of concentrations was different. The bacterial unknowns (*B.g.* and *E.h.*) had nominal concentrations of 10^2, $10^{2.5}$, 10^3, 10^4, or 10^5 cfu/mL The MS2 unknowns had nominal concentrations of 10^4, 10^5, 10^6, or 10^7 pfu/mL. During the

JFT4, we did not take an instrument to detect proteins, so we did not detect ovalbumin in unknowns. We were given 32 unknown samples per day, five days per week, for two weeks. Results had to be submitted at the close of each day for scoring.

One item of particular interest is the influence of Poissonian sampling statistics on the detection of *E.h.* unknowns. Figure 6 is a plot of the number of cycles of PCR that we had to run to call a positive versus the nominal concentration of unknowns, as revealed to us after the field trials ended. While essentially every unknown containing *E.h.* with nominal concentrations of $10^{2.5}$ cfu/mL or higher was identified and quantified, the roughly 5-µL sample size combined with the typical, actual concentration of about $2X10^2$ cfu/mL for unknowns with nominal concentrations of 10^2 cfu/mL meant that roughly 1/e of the time there was no DNA target in the PCR reaction, and produced, therefore, a false negative. This is to be expected when the PCR reaction is working well; a single piece of target DNA can be detected via PCR. One needs, however, to perform sample preparation that produces *on average* 10 pieces of target DNA in the PCR reaction in order to have 99.99% certainty of getting positive data on any particular PCR run. Although these data do reflect the sampling statistics, when we presented these data to a National Academies/National Research Council panel on 30 March 2000, a colleague of ours was in the audience and became inspired to do a thorough demonstration of the effect of Poissonian sampling on real-time PCR assays. The assays that we had performed at the JFT4 that demonstrated the effects of Poissonian statistics were ones in which the unknowns of *E.h.* contained living bacteria (which are difficult to quantify) at a concentration of $2X10^2$ cfu/mL. Each unknown had been prepared individually over a 2-week period, and were run in a variety of hand-made PCR chambers, each with slightly different thermal-cycling characteristics from the others. Our colleague repeatedly sampled a stable solution of DNA (not live organisms) at

4 X 10^3 targets/mL, including all the of the necessary PCR reagents, and ran every reaction in the same chamber. This produced a very clear Poissonian distribution of results for the real-time polymerase chain reaction[25]. After the JFT4, Dr. Belgrader had the time to optimize the PCR for *E.h.* using the ANAA, and these results have also been published[24]. The interest in portable PCR instrumentation led to a prototype hand-held device, known as the HANAA[25] and, eventually, this was commercialized[26].

Lessons learned from Field Trials

Before the JFT3, neither flow cytometers nor PCR instruments had been viewed as "contenders" for field use, either because of issues associated with lack of ruggedness or because of the issue of time to perform the assays, respectively. In 1996, for the JFT3, we clearly demonstrated that it was possible to design a transportable, high-performance flow cytometer that could be deployed rapidly. We also demonstrated the power of a flow cytometer for performing immunoassays in the field. We discovered, in the process, that the reagents, themselves, tended to limit the performance of the assays that we ran on the flow cytometer - cross reactivity and autofluorescence bounded the lower limits of sensitivity. Specifically, we had invented a new method for collecting the scattered light in our flow cytometer that used the flow stream itself as an optical waveguide[15], and this was what enabled us to build such a high-performance flow cytometer that was also transportable and field-deployable[16]. We showed that the flow-stream waveguide provided higher optical throughput with less background noise than the best commercial, non-transportable flow cytometers[17] that were intended to remain

on lab benches. However, when we ran the assays, we found that the limit of detection using directly-labeled antibodies and triggering on the elastic scattering properties of the bacteria was roughly 30 to 40 bacteria per milliliter, whether we used the flow-stream-waveguide flow cytometer or our best bench-top commercial flow cytometer. We also learned being able to run each sample through the flow cytometer in a minute or less was of particular value when dealing with many samples, day after day. Nonetheless, it was also clear that the single-target, single-reagent immunoassays that we used in the JFT3 would not scale to a system that needed to test for the presence of dozens or even hundreds of possible agents – we had to move to multiplexed assays, in the future.

In 1998, for the JFT4, we also demonstrated that PCR could operate in a timely manner, based on the fluorogenic Taqman™ assays. During the JFT3, we were also taught that even a high-performance instrument could be hampered by ineffectual assays. Another important lesson for all concerned was that PCR assays, themselves, do not measure viability of bacteria. This confounded some of the assays that we ran on the unknowns, since the labware that was used to prepare unknowns had been washed and autoclaved, but still occasionally contained enough residual DNA from the *E.h.* unknowns to generate weak positive signals with PCR, but did not grow any colony-forming units of *E.h.* from overnight culture. Thus, of the 224 unknowns during the JFT4 that were negative for the overnight *E.h.* culture assay, PCR showed 20 of these contained enough residual DNA to detect.

Autonomous systems

Robin Miles led a team of scientists and engineers that created the first APDS, shown in Figure 7, which was a single-assay

format and employed the Microcyte® flow cytometer. This system was taken to the wind tunnel at the Pacific Northwest Laboratory (special thanks to Drs. Mark Kingsley and Gary Dennis at PNL for inviting us and running these tests in their wind tunnel) in 1999 and tested against challenge releases of *B, atrophaeus*, (referred to as "*B.g.*", from its older name "*B. globigii*") the anthrax simulant. The following is taken from the report of Robin Miles:

> The autonomous detector consisted of a Research International SASS2000 aerosol collector coupled with a custom fluidic system built at LLNL using many off-the-shelf components, coupled with a Microcyte® flow cytometer. A central computer instructed the aerosol collector to collect sample, the sample preparation to meter and mix the sample with reagents, and the flow cytometer to check for binding of the antigen to the antibodies. The PNL facility consisted of an enclosed wind tunnel with the capability to inject particles into the flow stream via fluidized bed or nebulization in sugar solution or in methanol. The APDS I system was installed at PNL on May 26. On May 27, a 10-hour run was performed followed by a 2-hour run. Samples were collected every 15 minutes during these runs. A 6-hour run was conducted on May 28. In the course of these runs, all three methods for dispersion of *B.g.* were tested. The system correctly called positives down to the 300 ACPLA [agent-containing particles per liter of air] level.

Following the successful field test of the APDS I system, Drs. Langlois, Colston, McBride, and Dzenitis each led the multi-year effort for a multiplexed APDS II that included both immunoassays

and PCR assays.[28, 29] The APDS II sported major improvements in its design over that of the APDS I:

- A new, power-efficient pre-collection fractionator was invented[30] and installed, upstream of the wetted-wall cyclone aerosol collector
- Global FIA, Inc., precision multi-position valves and syringe pumps were incorporated in order to meter the sample and reagent solutions
- A Luminex Flow Cytometer was incorporated, enabling the use of multiplexed immunoassays via its “liquid array” of color-coded beads
- Disposable, closed-ended polypropylene tubes that had been used on the ANAA were replaced with a continuous fluidic tube that was captured inside of the sleeve-based thermal cycling chamber[18] for PCR for flow-through operation

The details of operation of the flow-through PCR system, including the multi-position valve, can be found elsewhere[31].

The system is shown in Figures 8 and 9.

Acknowledgment

The author wishes to thank our colleagues Drs. Gary Long, Tom O'Brien, and William Nelson, who provided the PCR and immunoassay reagents that we employed at the Field Trials, plus the large team of scientists and engineers who both made the instrumentation and also executed the demanding logistics of field work: Phillip Belgrader, John Dzenitis, Bill Colston, Mary McBride, Richard Langlois, Les Jones, Fred Milanovich, William Benett, Jim Richards, Paul Stratton, Kodumudi Venkateswaran, Shanavaz Nasarabadi, Steve Brown, Paula Kato, Phoebe Landre, and Don Masquelier.

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Figure Captions

Figure 1. Photograph of LLNL personnel during the laboratory portion of the Joint Field Trials 3, held in the “old Baker Laboratory” at Dugway Proving Grounds in Sept-Oct 1996. Pictured, from left to right are Phoebe Landre, Steve Brown, Raymond Mariella Jr., Paula Kato, Richard Langlois, and Don Masquelier. The miniFlo flow cytometer is just out of view, to the right.

Figure 2. Photograph of “miniFlo, the flow cytometer that we took to JFT3 in 1996, which was not visible in Figure 1.

Figure 3a. Plot of concentration of unknowns containing *Bacillus globigii* versus their official concentrations, as revealed after the termination of the Joint Field Trials 3. The ordinate is referenced to the concentration of standard/calibration beads that we included in the assay. An ordinate of 1 indicates a concentration of 5000/mL. The abscissa is nominal concentration of unknowns, expressed as colony-forming units/mL (cfu/mL). For example, by our measurements, the unknowns with a nominal concentration of 10^3/mL appeared to fall between 1.5 and 2 X 10^3 cfu/mL.

Figure 3b. Plot of concentration of unknowns containing *Erwinnia herbicola* versus the official concentrations (cfu/mL), as revealed after the termination of the Joint Field Trials 3. The procedure was the same as in Figure 3a.

Figure 4a. Plot of concentration of unknowns containing the retrovirus MS2 versus their official concentrations, as revealed after the termination of the Joint Field Trials 3. The ordinate was derived by a

dose-response curve that we established using standard concentrations of MS2. The abscissa is the nominal concentration of the MS2, expressed in plaque-forming units/mL (pfu/mL). The non-linear behavior of the curve for 10^5 pfu/mL was due to intrinsic fluorescence of the capture beads.

Figure 4b. Plot of concentration of unknowns containing the protein ovalbumin versus their official concentrations, as revealed after the termination of the Joint Field Trials 3. The ordinate was derived by a dose-response curve that we established using standard concentrations of ovalbumin. The abscissa is the nominal concentration of the ovalbumin, expressed in ng/mL. Again, the intrinsic fluorescence of the capture beads limited the performance at the lowest concentration of unknowns.

Figure 5. Photograph of some of the LLNL personnel during the laboratory portion of Joint Field Trials 4, held in the “new” Baker Laboratory of Dugway Proving Grounds, January 1998. Pictured from left to right are Kodumudi Venkateswaran, Phillip Belgrader, and Raymond Mariella Jr. The “Advanced Nucleic Acid Analyzer” or “ANAA” is visible on the laboratory bench.

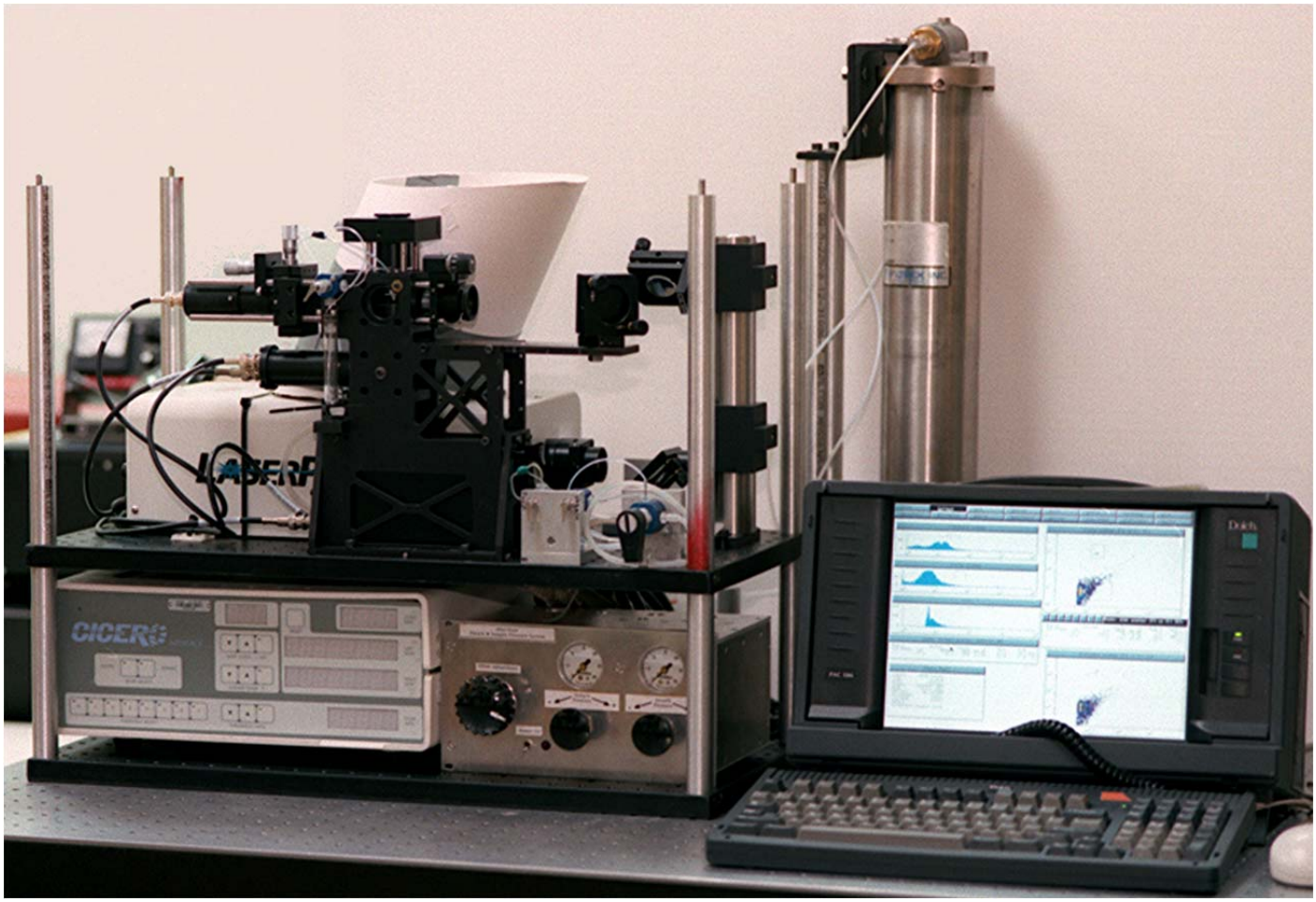
Figure 6. Plot of the threshold cycles for the PCR *E.h.* assay that we measured for the 100 unknowns versus the concentrations, based on culture assays, as revealed after the termination of the Joint Field Trials 4. Since PCR determined the presence of DNA and the culture assay measured live bacteria, there were some differences. The most notable differences were that some of the unknowns that were nominally blank for *E.h.* had sufficient residual Eh DNA so that the PCR was able to detect its presence. This was true for 20 out of 224 samples.

Figure 7. Photograph of the APDS I, being set up by Les Jones at the PNL wind tunnel, 1999.

Figure 8. Photograph of the APDS II, deployed in the Washington, DC, Metro.

Figure 9. Photograph of the APDS II, with its access door open, displaying, from top to bottom, the reagent and liquid-waste-storage containers, the control computer, the fluidic system, and the Luminex flow cytometer.

Figures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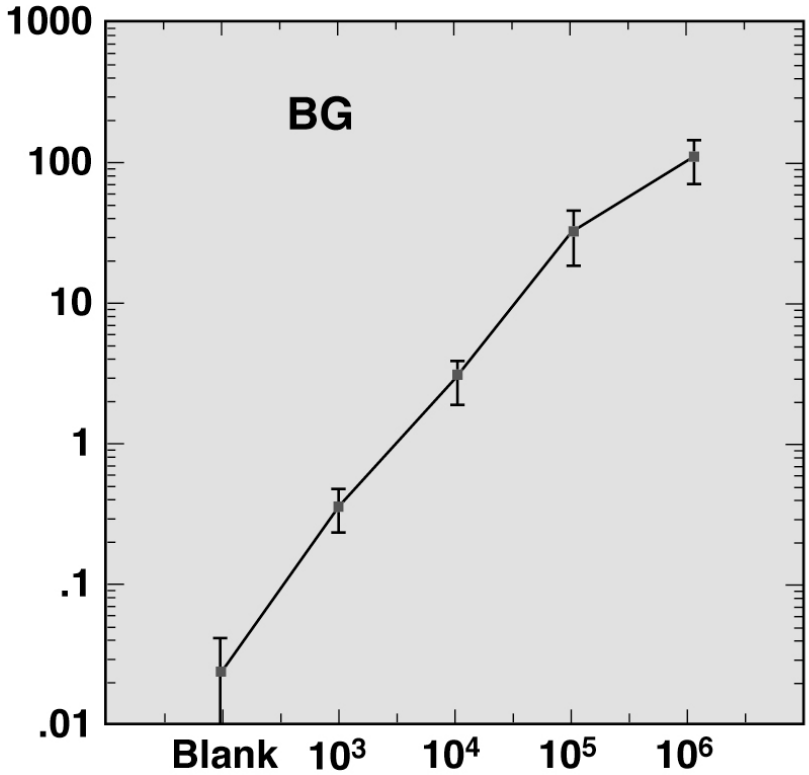
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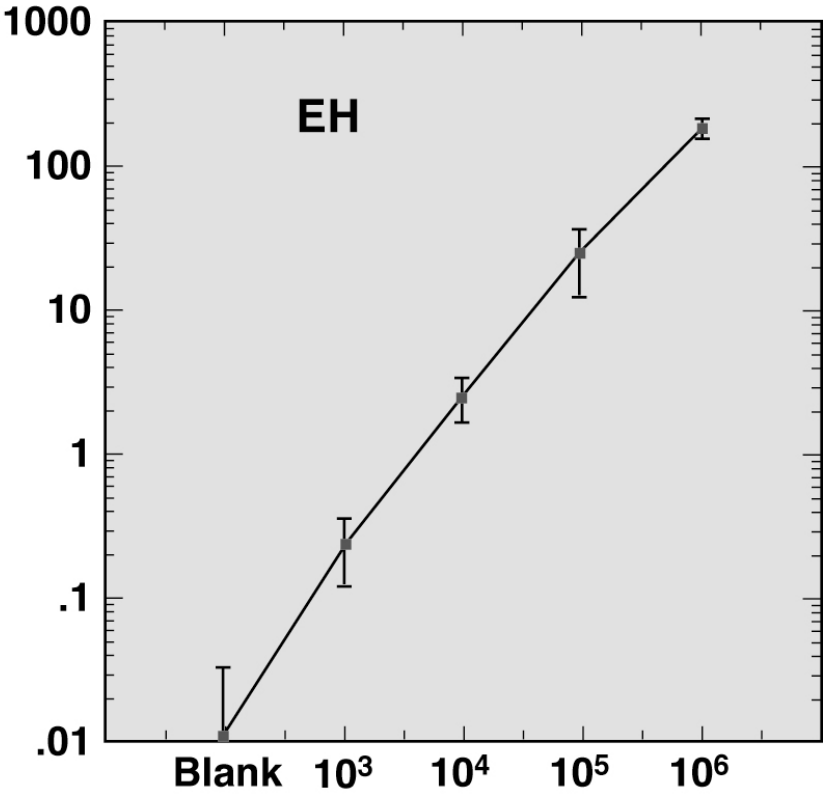
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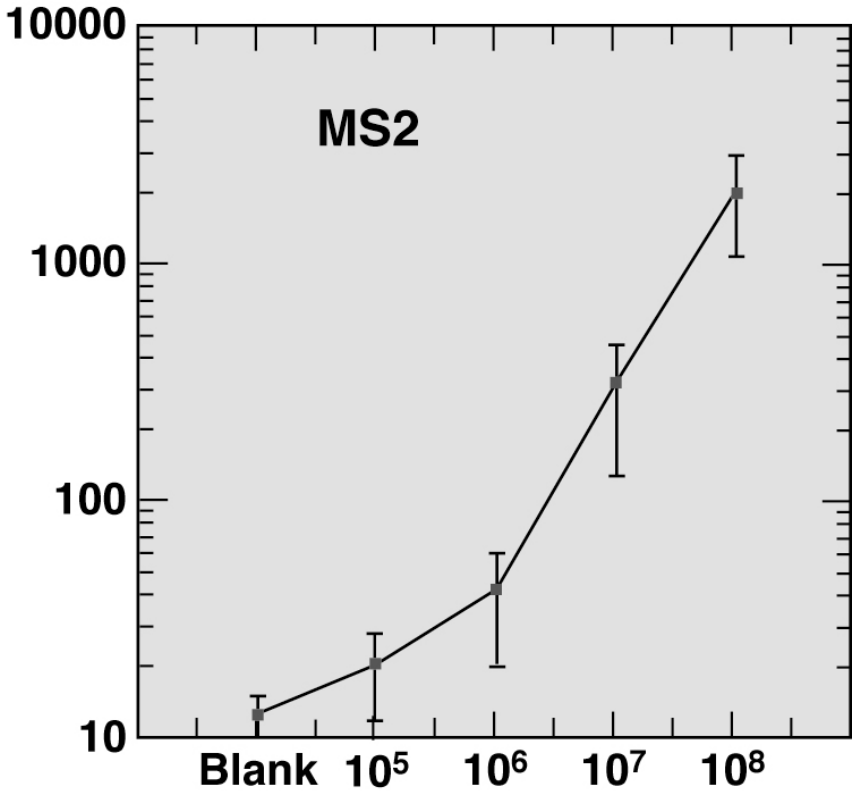
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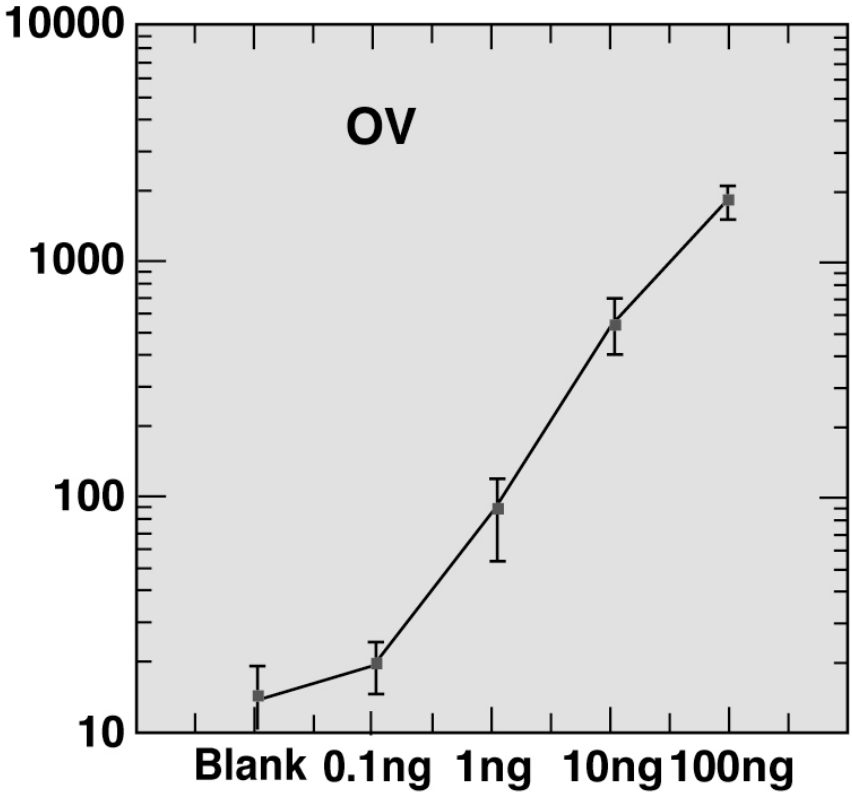
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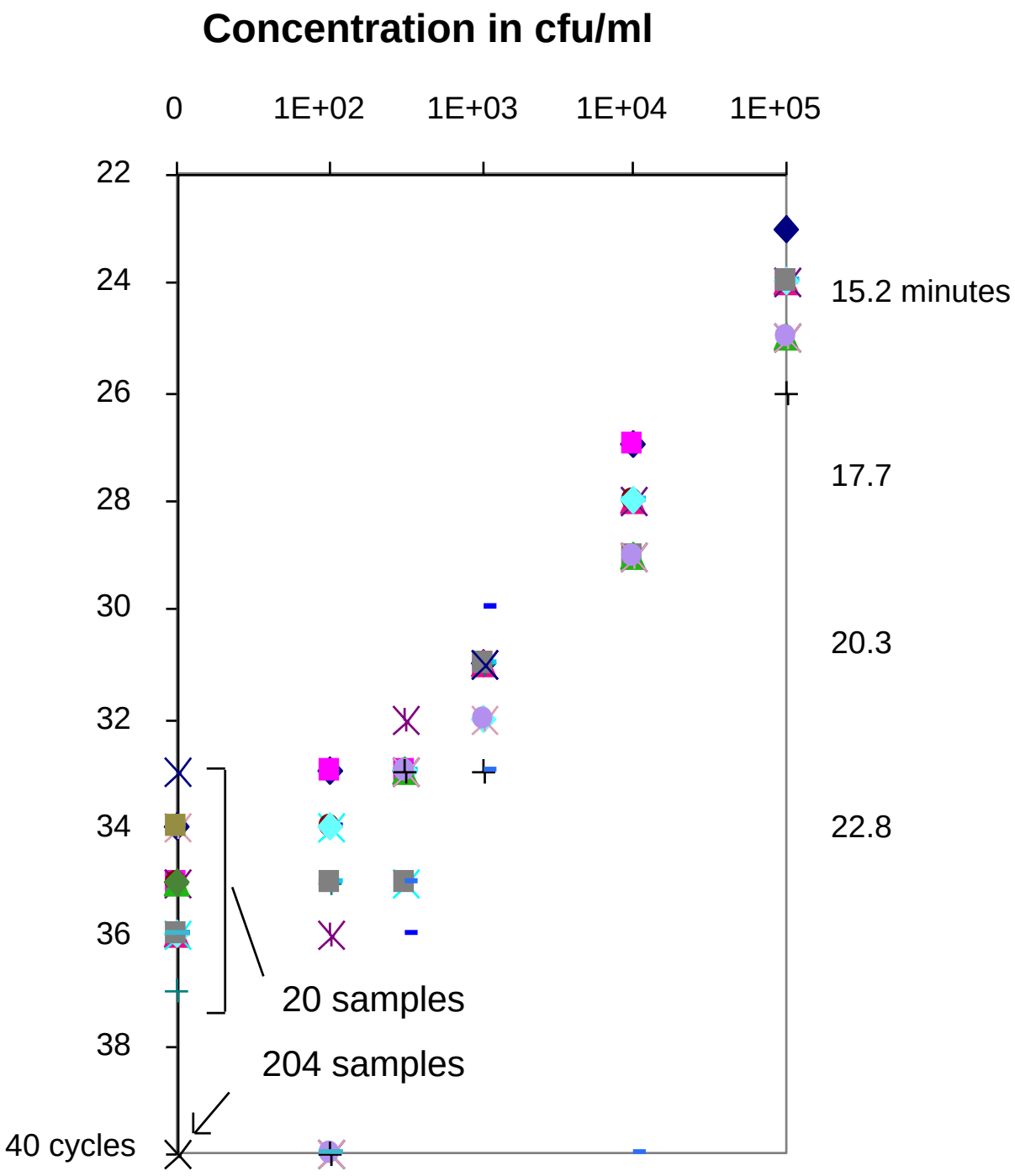
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17.7
20.3
22.8
20 samples
204 samples


Luminex 100